

# Biogeographical affinities of the aquatic community of Refugio Cave, a newly discovered *Astyanax* cave

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Academic editor: Maria E. Bichuette | Received 16 February 2023 | Accepted 1 July 2023 | Published 21 September 2023

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<https://zoobank.org/74CA7568-1A4F-4067-A507-98EBDCDF8341>

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**Citation:** Espinasa L, Pech A (2023) Biogeographical affinities of the aquatic community of Refugio Cave, a newly discovered *Astyanax* cave. Subterranean Biology 46: 77–86. <https://doi.org/10.3897/subtbiol.46.102043>

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## Abstract

Pachón cave in the Sierra de El Abra, in Northeast Mexico, stands out as hosting the world's most widely studied cavefish population – with over 500 scholarly articles published about the population. Refugio Cave was recently discovered in the El Abra region. This cave hosts the mysid cave shrimp *Spelaeomysis quinterensis* and the blind cave tetra fish, *Astyanax mexicanus*. This study aims to understand how the aquatic community of Refugio Cave is related to other cave populations in the area. For this purpose, the Histone *H3* gene of mysid shrimps and the *OCA2* gene that confers albinism in *Astyanax* fish was sequenced. Results support that the Refugio and Pachón aquatic communities, which are only 4.5 km away apart, are closely related. Thus, the Refugio Cave population may contribute to better understand the evolutionary history of such an important population and, perhaps, help with Pachón's cavefish conservation.

## Keywords

*Astyanax*, Pachón Cave, Sierra de El Abra, stygobite, troglobite, troglomorphy

## Introduction

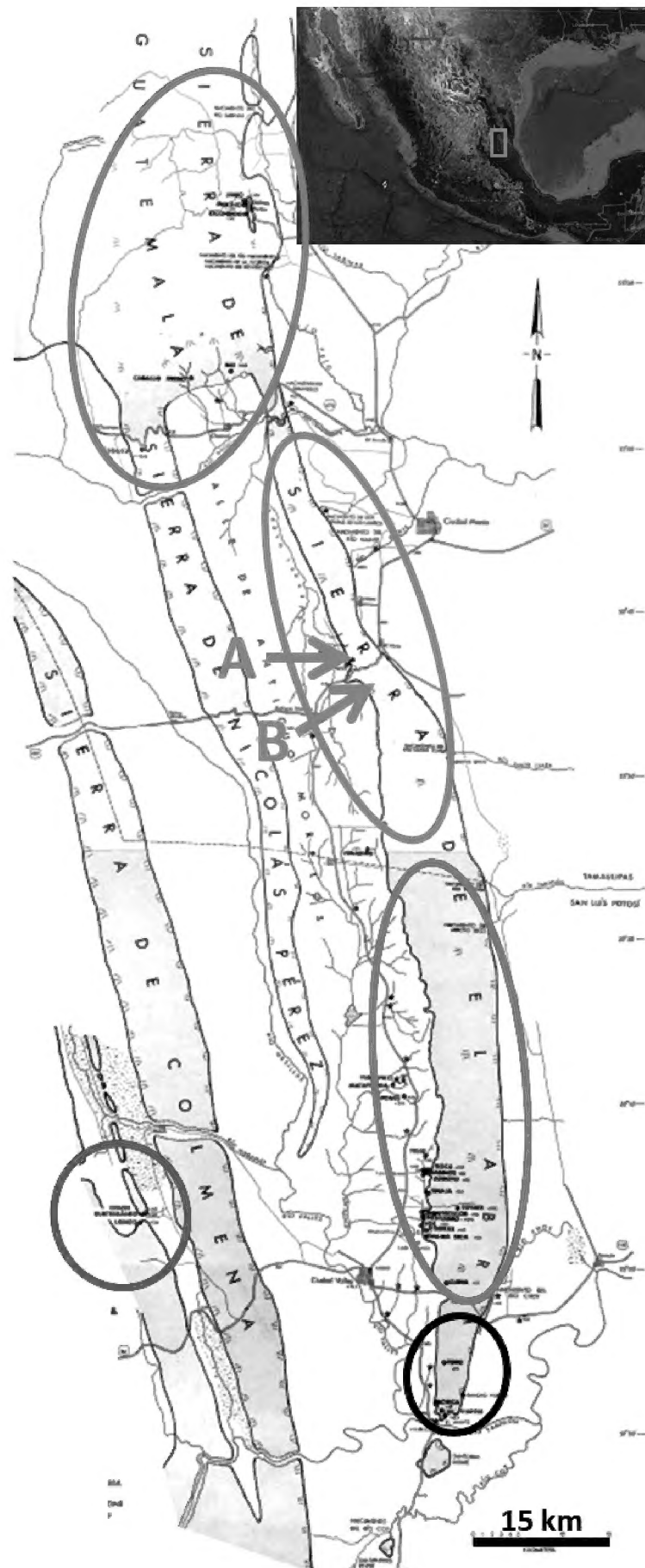
The El Abra region in northeast Mexico stands out as one of the most important places for cave biology studies (Elliott 2018). The recently discovered Refugio Cave (Miranda-Gamboa et al. 2023) hosts the blind cave morph of the tetra fish, *Astyanax mexicanus*, and the mysid cave shrimp *Spelaeomysis quinterensis*. Through the sequencing

of genetic markers in these two species, this study attempts to understand how the aquatic community of Refugio Cave is related to other cave populations in this area.

The blind Mexican tetra of genus *Astyanax* is ranked among the influential model systems in evolutionary developmental or EvoDevo biology (Jeffery 2001, 2012). It has also become the main contributor to understanding the genetic and developmental controls of troglomorphic features. These cavefish have a conspecific surface-dwelling morph that lives in nearby surface streams throughout most of México. Both surface and cave morphs remain inter-fertile, making the species complex and well-suited for experimental manipulations (Jeffery 2012; Elipot et al. 2014). Among multiple *Astyanax* cavefish populations, those from Pachón cave stand out as the world's most widely studied cavefish population (Keen et al. 2015). The genome from this population was the first cavefish to be sequenced (McGaugh et al. 2014; Warren et al. 2021). With fish from this population, a seminal study showed the central role of the lens in cavefish eye degeneration (Yamamoto and Jeffery 2000). The recently discovered Refugio Cave (Miranda-Gamboa et al. 2023) is only 4.5 km from Pachón cave and, thus, the closest among all caves with cavefish to this emblematic locality.

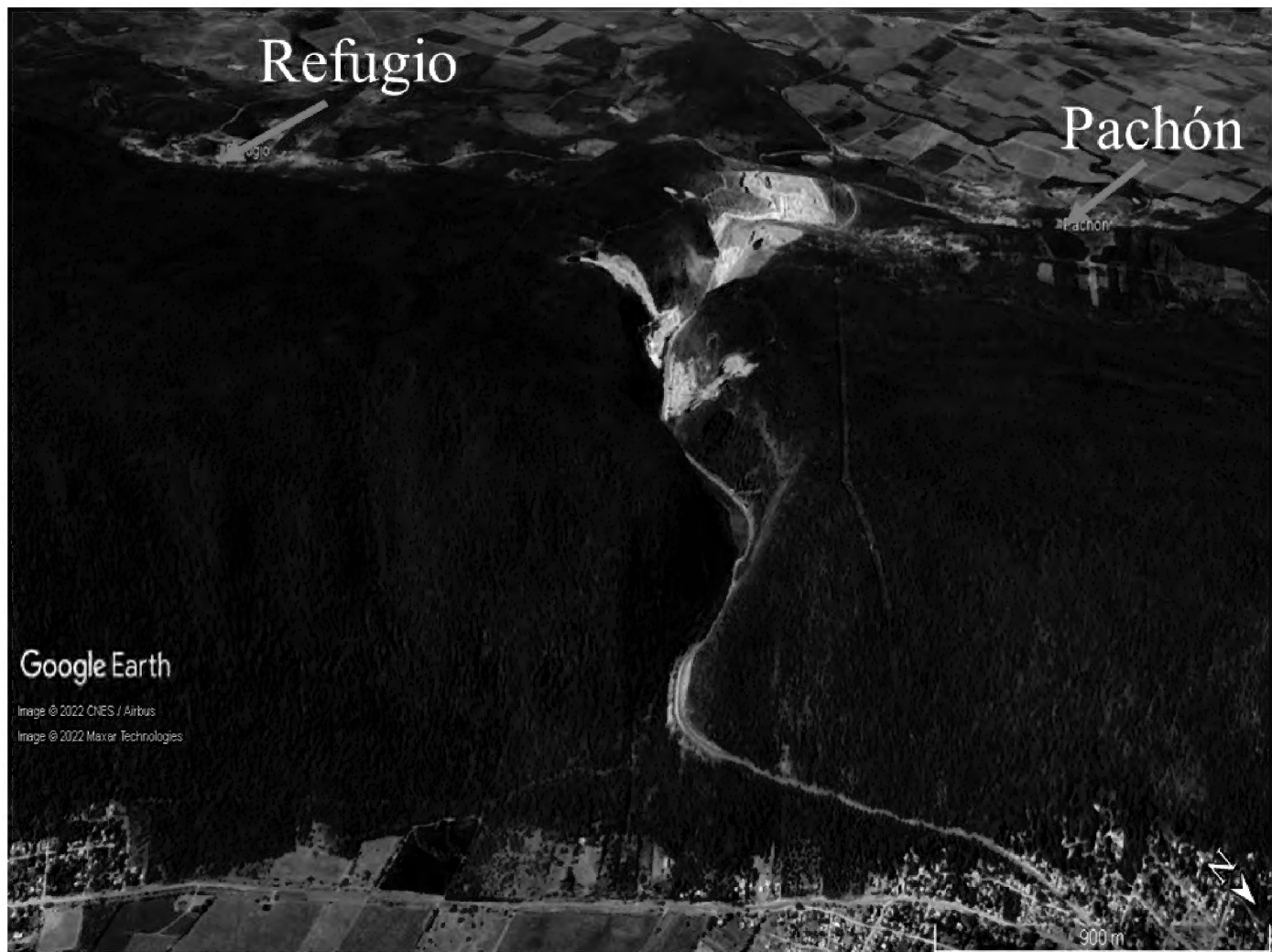
El Abra caves are also inhabited by the mysid cave shrimp *Spelaeomysis quinterensis*. Based on Histone *H3* DNA sequences of the mysids, mitochondrial DNA from *Astyanax*, and the underground hydrogeology of the region, Kopp et al. (2018) identified five biogeographic zones for the El Abra region: Sierra de Guatemala, Northern Sierra de El Abra, Central Sierra de El Abra, Southern Sierra de El Abra, and Micos (Fig. 1). The caves within these biogeographical zones share hydrologic connections (Espinasa and Espinasa 2016). It is likely that throughout their evolutionary history, cavefish populations within these zones exchanged individuals, as reflected by gene flow and population structure studies (Bradic et al. 2012). All biogeographical zones have multiple caves with *Astyanax* cavefish (Elliot 2018). The single exception is the northern Sierra de El Abra area. Pachón cave stands alone as the single described cavefish population within this biogeographic zone.

Is the “Refugio” cave within the northern Sierra de El Abra biogeographic zone, and thus, its *Astyanax* and mysid shrimp share genetic and evolutionary proximity to the Pachón community? Its geographical proximity would suggest that they could be genetically closely related. But further geographical analyses show there may be barriers for the two populations to exchange individuals. While most caves in central Sierra de El Abra are sumideros where the water goes in, and thus, the caves can share a common aquifer, on the contrary, both Pachón and Refugio Caves are independent springs with water coming out of the cave system, and do not share a common aquifer (Miranda-Gamboa et al. 2023). They would have to connect at the headwaters, and the 4.5 km distance between the two caves makes it a considerable barrier. Geology also has challenges. Between Pachón and Refugio caves, there is the remnant of a fossil canyon, which is now used by Highway 85 to Cd. Mante. This fossil canyon is almost 100 m deep (Fig. 2). Espinasa and Espinasa (2016) have argued that as tectonism elevated the El Abra region and layers above the limestone eroded away, the rivers changed their course. Around one to four mya, Río Comandante made the aforementioned canyon, and around 0.75 to three mya, as tectonism continued to elevate the area, the Río Comandante changed its route



**Figure 1.** Map of the El Abra region, showing the five biogeographic zones proposed by Kopp et al. (2018) and supported by both *Astyanax* and mysids in this study: Sierra de Guatemala (yellow), Northern Sierra de El Abra (Red), Central Sierra de El Abra (blue), Southern Sierra de El Abra (Black), and Micos (green). **A** Pachón cave **B** Refugio Cave. Before this study, Pachón was the only known cave to harbor cavefish in the northern Sierra de El Abra. Map modified from Mitchell et al. (1977).

farther north to its current position at Servilleta canyon (Espinasa and Espinasa 2016). Several of the fossil canyons in the Sierra de El Abra have been shown to be considerable biogeographical barriers for the dispersal of aquatic organisms (Espinasa et al. 2020).



**Figure 2.** Between Pachón and Refugio Cave (22°34'28.50"N, 99°01'30.74"W), there is a deep fossil canyon. This canyon may be a barrier for active water connection between the two caves. Distance between Refugio and Pachón caves is 4.5 km.

While there may not be an active hydric connection between the two caves nowadays, biological communities could still be very closely related. The purpose of this study is to determine if the aquatic community inhabiting Refugio Cave is closely related to the one in Pachón. The alternate hypothesis is that the aforementioned canyon is a biogeographical barrier, and the Refugio community belongs within the Central Sierra de El Abra biogeographic zone. Two genetic markers will be used, the *Oca2* gene for *Astyanax* and the Histone *H3* for Mysid shrimps.

Most *Astyanax* cave populations are depigmented, but the Pachón population evolved albinism independently due to a unique mutation in the Ocular and Cutaneous Albinism Type II (*Oca2*) gene (Protas et al. 2006). Pachón fish have a base change (guanine instead of adenine) in exon 13 at position 1252. This unique allele can distinguish northern cavefish from the central Sierra de El Abra populations.

Mysid shrimp in central Sierra de El Abra derive from a separate lineage different from the rest of the Sierra de El Abra and Sierra de Guatemala populations (Kopp et al. 2018): When analysing the H3 marker, Pachón's mysids differ from Sierra de Guatemala populations by 7 bp (2.1%) and from central Sierra de El Abra by 31–36 bp (9.4–10.9%). The H3 sequence can be used to differentiate northern Mysids from the central Sierra de El Abra populations.



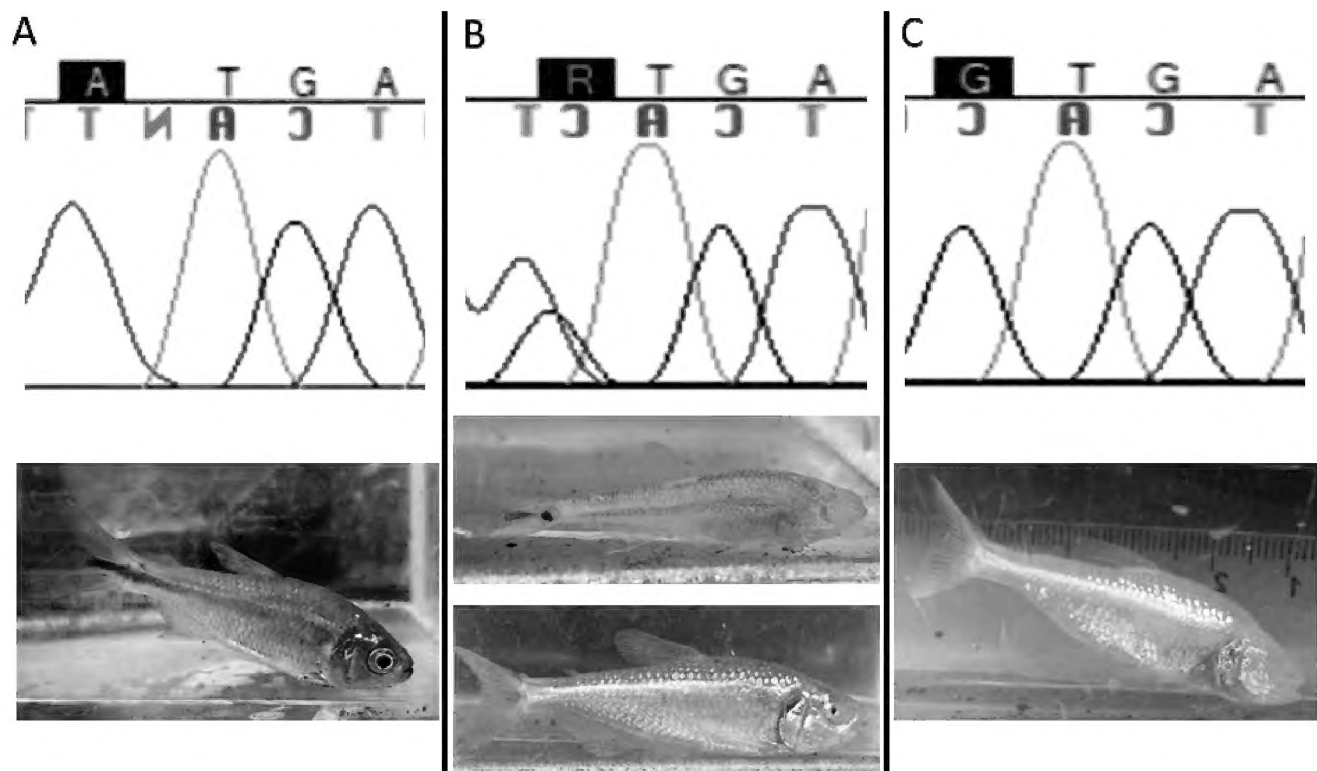
## Methods

*Astyanax mexicanus* samples from Refugio Cave (N=11) were the same used in Miranda-Gamboa et al. (2023). Samples of *Spelaeomysis quinterensis* were collected from Refugio Cave (N=3) and Piedras Cave (N=2) in Central Sierra de El Abra. To test if the *Astyanax* Refugio population is closely related to the Pachón population, we examined if the specimens from Refugio Cave have the *Oca2* allele with the single nucleotide polymorphism (guanine instead of adenine) within exon 13 at position 1252, found in Pachón cavefish. Surface and Pachón cavefish sequences were obtained from Protas et al. (2006), Espinasa et al. (2014), and GenBank # DQ232591. To test the same hypothesis for the mysid shrimp, we used the Histone *H3* marker and compared against available sequences from Caballo Moro cave in the Sierra de Guatemala, Pachón from Northern Sierra de El Abra, Tinaja cave in Central Sierra de El Abra, and Chiquitita cave from Southern Sierra de El Abra (Kopp et al. 2018; GenBank # MH422492–MH422494). The *H3* marker has been shown to differentiate among mysid populations inhabiting different biogeographical zones of El Abra (Kopp et al. 2018). For the collection of cavefish specimens, permission was obtained from the competent Mexican authorities (SEMARNAT SGPA/DGVIS/03334/22). All the fish collected were kept alive in the laboratory of Patricia Ornelas-García, IBUNAM, for breeding in captivity, which can serve as a stock for future studies.

Genomic DNA was extracted using Qiagen's DNEasy Tissue Kit by digesting a fin clip or a leg in lysis buffer. A polymerase chain reaction (PCR) amplification of a 66 bp *Oca2* gene fragment was performed using the primers 5'-GCGTATCAGGTGTCCA-GAGG-3' and 5'-AGAGCATCATGGTGGTCACA-3' with an annealing temperature of 55 °C, as in Espinasa et al. (2014). For *H3*, the following primer pair was used H3aF (5' ATGGCTCGTACCAAGCAGACVGC 3') and H3aR (5' ATATCCTTRG-GCATRATRGTGAC 3') with an annealing temperature of 55 °C, as in Espinasa et al. (2007). PCR amplicons were purified using the QIAquick PCR Purification Kit by QIAGEN and sent to Elim Biopharmaceuticals for sequencing. Chromatograms obtained from the automated sequencing were read, and contigs were made using the sequence editing software Sequencher™ 3.0. External primers were excluded from the analyses. Sequence identity was confirmed through BLAST analyses. Sequences were aligned with ClustalW2. For mysid's *H3* sequences, the total number of bp differences among populations were counted with Sequencher™ 3.0. For *Astyanax*'s *Oca2*, chromatograms were visualized at base position 1252 to determine if they were homozygous for guanine or adenine, or if they had a double peak – which is characteristic of heterozygous genotypes.

## Results

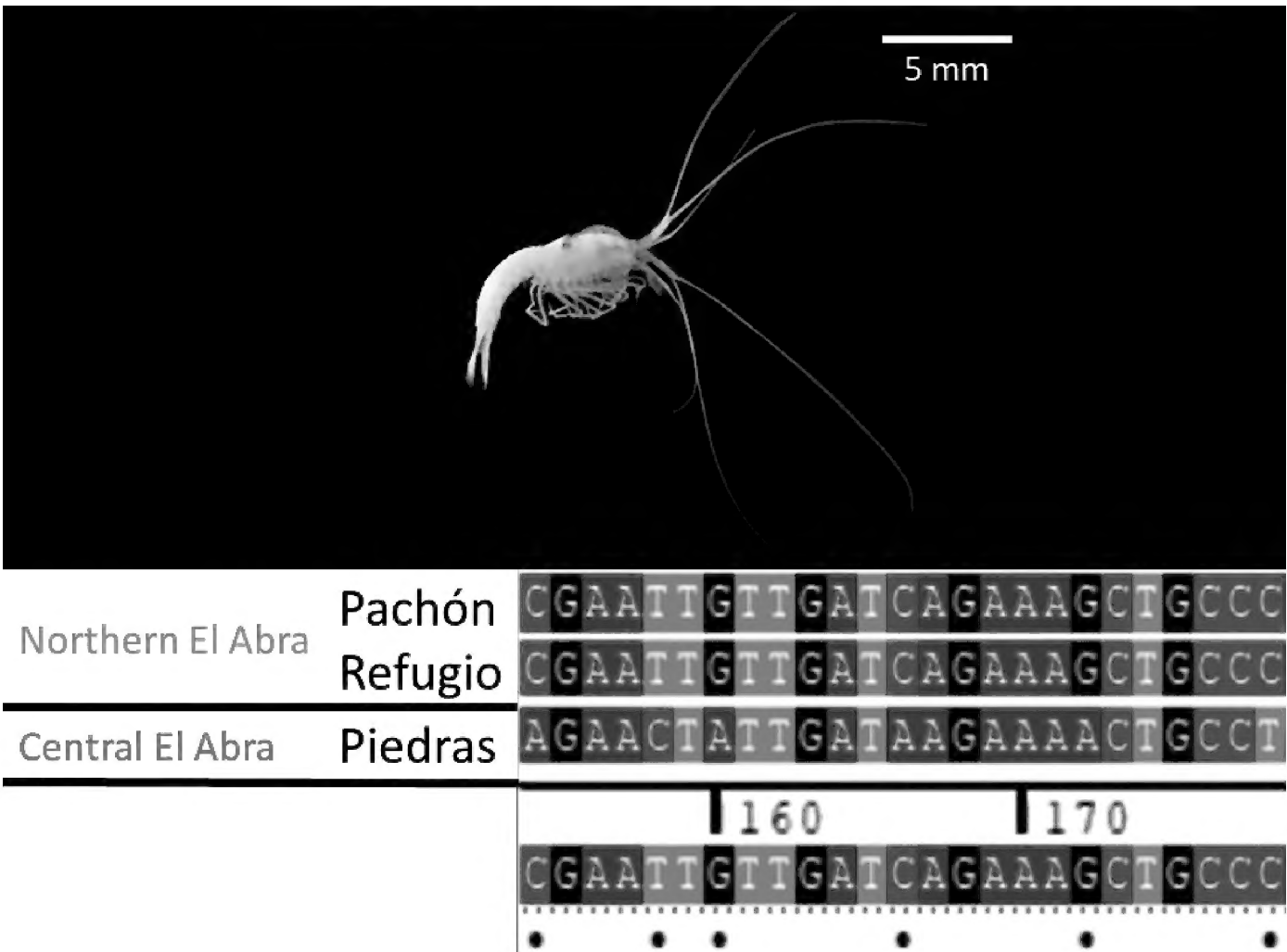
The Refugio Cave entrance is at 229 masl and at the same base level as the nearby ephemeral surface stream that flows only during the rainy season. Thus, it is likely



**Figure 3.** Chromatogram of exon 13 at position 1252 of the *Oca2* gene in Refugio Cavefish. **A** epigeomorph fish from Refugio Cave have an adenine (green hump), just like surface fish **B** some hypothesized hybrids that show either a dark caudal spot or a dark retina, and thus are not phenotypically albino albeit being depigmented, were heterozygous, showing a distinct double green and black hump for adenine and guanine **C** the most troglomorphic fish of Refugio Cave were homozygous for guanine (Black hump), which is in the albino allele reported only for Pachón cavefish among all Sierra de El Abra populations. Fish photos modified from Miranda-Gamboa et al. (2023).

that every rainy season surface fish have direct access to El Refugio Cave (Miranda-Gamboa et al. 2023). This is unlike Pachón cave, where the entrance is at an altitude of 203 masl, perched on a steep hill with the base level of the valley at 175 masl, thus, with a strong geographic barrier preventing the influx of surface fish. As reported in (Miranda-Gamboa et al. 2023), Refugio Cave hosts an *Astyanax* mixed population of epigeomorph fish fully pigmented, with large eyes, identical to surface fish (Fig. 3A), hybrids with either some type of eye and/or pigment (Fig. 3B), and specimens with no pigment or external remnants of eyes that look as troglomorphic as Pachón cavefish (Fig. 3C). Results also show the presence of highly depigmented individuals with eyes, and individuals with pigment but reduced eyes (Fig. 3B) as would be expected if there was introgression between the surface and cave morphs.

DNA sequences corroborated this assumption (Fig. 3). Presumptive hybrids with different degrees of eye and pigment regression were heterozygous (N=6) at base position 1252 of the surface *Oca2* allele, the most epigeomorph fish (N=1) had the adenine found in surface fish, and the most troglomorphic fish had the guanine of the cave allele (N=4). Most relevant, these results show that some individuals of Refugio Cave have the same SNP in the *Oca2* gene as the Pachón cavefish. This SNP has not been reported in other Sierra de El Abra cavefish populations. Thus, suggesting a close relationship between Pachón and Refugio *Astyanax* populations. This information indicates that the Refugio population belongs to the Northern Sierra de El Abra biogeographical zone.



**Figure 4.** Mysis shrimp sequences of the Histone *H3* gene from Refugio Cave were only two bp (0.6%) different from Pachón and 34 bp (10.3%) from the Central Sierra de El Abra cave population of Piedras. Sabinos and Tinaja cave populations, also from Central El Abra, had identical sequences (not shown) to Piedras. This suggests *Spelaeomysis quinterensis* in Refugio Cave belong within the Northern Sierra de El Abra biogeographical zone and closely related to Pachón.

Mysis DNA results support that this may be true for the *Astyanax* cavefish and the entire aquatic community. The *H3* fragments were 328 bp long (GenBank # MH422492–MH422494). Mysis Refugio sequences were only two bp (0.6%) different from Pachón, 7 bp (2.1%) from Sierra de Guatemala, and 34 bp (10.3%) from the Central Sierra de El Abra cave populations of Sabinos, Tinaja, and Piedras, which had identical sequences (Fig. 4). Thus, suggesting that both aquatic species, *Astyanax* and *Spelaeomysis quinterensis* in Refugio Cave belong within the Northern Sierra de El Abra biogeographical zone (Fig. 1).

Discussion

Kopp et al. (2018) showed that the El Abra region has distinct biogeographic areas with partial barriers that affect evolutionary histories, creating evolutionary significant units for all members across distinct species of the aquatic cave community. For the Sierra de El Abra, three major biogeographic zones were delineated. A Northern, a Central, and a Southern zone. To reach this conclusion, they used hydrogeography, *H3*

DNA from the mysid Shrimp *Spelaeomysis quinterensis*, and mitochondrial DNA from *Astyanax mexicanus*. Support for such zones can also be found with nuclear markers of *Astyanax* (For ex., Bradic et al. 2012).

Our results from the mysid shrimp sequences of *H3* support that the Refugio Cave population is closely related to the Pachón population in the northern Sierra de El Abra, and very distantly related to the Central zone cave population of Sabinos, Tinaja, and Piedras (Fig. 4). Likewise, at least some Refugio *Astyanax* individuals have the same base change allele in exon 13 at position 1252 in the *Oca2* gene as in the Pachón population (Fig. 3). This allele has not been described in any other *Astyanax* populations in the Sierra de El Abra. Finally, Mitochondrial DNA sequences of Refugio *Astyanax* showed them to have the same haplotype found in the Pachón and surface populations, and not the one found in the Central Sierra de El Abra populations (Miranda-Gamboa et al. 2023). Taken together, these results suggest that the aquatic community at Refugio Cave belongs within the Northern zone (Fig. 1). It also supports that the 100 m deep remnant of a fossil canyon between the two caves (Fig. 2) – now used by Highway 85 to Cd. Mante – is not the barrier between the Northern and Southern biogeographic zones. This divide must lay further south, as proposed by Mitchell et al. (1977).

Fish from Pachón Cave stand out as the world's most widely studied cavefish population (Keen et al. 2015). Unfortunately, recent studies have suggested that its population may be declining and comprised of only a few hundred individuals (Legendre et al. 2022). With the information currently available, the Refugio Cave population appears to be the most closely related to Pachón in the entire El Abra region. Thus, the Refugio Cave population will help better understand current evolutionary forces as well as the evolutionary history of the Northern *Astyanax*. It could also serve as an alternative locality to conduct field studies, minimizing the impact on the Pachón population and helping with its conservation.

## Acknowledgements

Claudia Patricia Ornelas-García organized the expedition from which specimens from Refugio Cave were originally collected. This study was supported by the School of Sciences of Marist College. Thanks to all group members who participated in the field trip, particularly Ramses Miranda-Gamboa, Jorge Hernández-Lozano, and Jean Louis Lacaille. Proof-reading by Jordi Espinasa.

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